

## **Respiratory Avian Influenza A (H5N1) in Apparently Healthy Domestic Ducks (*Cairina moschata*) in Yogyakarta**

### **Avian Influenza (H5N1) Bentuk Pernafasan Pada Entok (*Cairina moschata*) Sehat Di Yogyakarta**

**Hastari Wuryastuti<sup>1</sup>, R. Wasito<sup>1</sup>**

<sup>1</sup>Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta  
E-mail : wuryastutyh@gmail.com

#### **Abstrak**

Virus influenza tipe A H5N1 telah mewabah pada unggas di Indonesia dan diduga secara sporadik telah menular ke manusia. Semua bukti yang ada menunjukkan, bahwa penyebaran utama virus AI di suatu daerah adalah melalui burung-burung liar. Bebek secara alamiah merupakan reservoir virus influenza tipe A dan kemungkinan menyebarkan virus AI dari peternakan satu ke peternakan yang lain melalui transfer secara mekanik feces yang infeksiif, dimana virus AI mungkin terkandung dalam konsentrasi yang cukup tinggi dan dalam kondisi tahan hidup dalam waktu yang relatif lama. Pada penelitian ini, kami laporkan hasil penelitian secara imunohistokimia dan molekuler dari 9 ekor entok yang diduga terinfeksi virus influenza tipe A secara alami. Dengan analisis molekuler RT-PCR, gen H5N1 terdeteksi dari parenkim paru, tetapi tidak dari pankreas maupun usus. Secara imunohistokimia *streptavidin biotin* digunakan monoklonal antibodi anti viral nukleoprotein, lokasi virus hanya ditemukan di dalam sel-sel endotelial pembuluh darah paru. Berdasarkan hasil penemuan ini, diduga bahwa entok dapat bertindak sebagai penyebar virus AI di lingkungan, tidak hanya melalui feces tetapi juga dapat melalui udara atau kontak langsung dengan sekresi pernafasan yang terkontaminasi. Selanjutnya disarankan, bahwa untuk aktifitas survei virus influenza pada unggas sangat penting untuk mengkarakterisasi virus AI pada bebek dan diperlukan persiapan yang cukup matang.

**Kata kunci:** entok, imunohistokimia, molekuler, antibodi monoklonal, penyebaran lewat udara

#### **Abstract**

Influenza virus type A H5N1 has become epidemic in poultry population in Indonesia and has been suspected sporadically to cross humans. All the available evidences suggest that the most common primary introduction of AI viruses into an area is by wild birds. The ducks form the reservoir of influenza type A viruses in nature and may spread AI virus from farm to farm by mainly mechanical transfer of infective feces, in which AI virus may be present at high concentrations and may survive for considerable periods. In the present paper, we report on the immunohistochemical and molecular investigations performed on nine ducks (*Cairina moschata*) naturally infected by influenza virus type A. The H5N1 gene was detected by molecular analysis of the reverse transcriptase-polymerase chain reaction in the parenchyma of the lungs, but neither in the pancreas nor the intestines. Immunohistochemical streptavidin biotin assay of monoclonal antibody anti nucleoprotein viral antigen also only corresponded to virus location within the vascular endothelia of the lung only. Our findings may suggest that the ducks provide an environment transmission of AI virus not only by a fecal route, but also by either an airborne route or direct contact with contaminated respiratory secretions. Moreover, we suggest that surveillance activities influenza viruses of the avian origin are critical for characterizing AI virus in the ducks and requires a high level of preparedness.

**Keywords:** ducks, immunohistochemical, molecular, monoclonal antibody, airborne route

## Introduction

During the end of 2003 Indonesia has been affected by an epidemic of highly pathogenic avian influenza due to an influenza virus of the H5N1 subtype. The disease has continued to spread within the poultry population in almost all of the provinces in Indonesia. And, even in the middle of July 2005, the first zoonotic transmission of an H5N1 caused 3 cases of human deaths in Indonesia raising concerns that this H5N1 virus could initiate the next human pandemic. To date, the human's death due to H5N1 infection is estimated to be 115 out of 141 cases in Indonesia. The production of severe respiratory disease and human mortality is unique to the Indonesia-origin H5N1 virus which was always suspected from the disease outbreak in chicken. The exact mode of transmission of this H5N1 virus to humans in Indonesia is still not known.

The virus causing avian influenza is an influenza virus type A which belongs to the family of *Orthomyxoviridae*. In wild birds and chickens around the world, influenza viruses type A carrying virus glycoproteins hemagglutinin (H) and neuraminidase (N) have been described. At present 16 H subtypes have been recognized (H1-H16) and nine neuraminidase subtypes (N1-N9) (Fouchier *et al.*, 2005). These viruses have been isolated from many animal species, including humans, pigs, horses, mink, marine mammals, and a wide range of domestic and wild birds (Murphy and Webster, 1996; Webster *et al.*, 1992).

Morbidity and mortality rates of influenza virus type A in chickens and wild birds are largely defined by species and virus, as well as age, environment, and concurrent infections (Easterday

*et al.*, 1997). Influenza virus type A infecting poultry can be divided on the basis of their pathogenicity. The very virulent viruses cause highly pathogenic avian influenza (HPAI) with mortality in poultry as high as 100%. Only the H5 and H7 subtypes are considered to be highly pathogenic in avian species having shown pantropic dissemination within the host (Bosch *et al.*, 1981; Kawaoka *et al.*, 1987). Other influenza viruses type A cause a much milder disease called low pathogenic avian influenza (LPAI). Clinical signs are much less evident or even absent and mortality is much lower.

Influenza virus type A H5N1 has circulated worldwide (Alexander, 2000; Alexander, 2001). In addition to avian species, wild birds, predominantly ducks, geese and shorebirds, form the reservoir of influenza type A viruses in nature. The viruses are distinct in their ability to infect cells lining the intestinal tract of birds and are excreted in high concentrations in their feces (Murphy and Webster, 1996; Webster *et al.*, 1992). Influenza virus type A is generally nonpathogenic in wild birds; it sometimes causes significant morbidity and mortality upon transmission to other species, including domestic birds and mammals (Murphy and Webster, 1996; Webster *et al.*, 1992). In the year 2000, for the first time, Gs/Gd/96-like viruses were repeatedly isolated from the ducks, and, by year-end, reassortant H5N1 viruses containing internal gene segments from other aquatic avian viruses were isolated from a goose and a duck (Guan *et al.*, 2002).

In the present report, during the bio-surveillance studies of influenza virus type A in avian species in Yogyakarta, Central Java, Indonesia we isolated influenza virus type A H5N1 from nine apparently healthy domestic ducks (*Cairina*

*moschata*). Interestingly, molecular analysis of the H5N1 gene and immunohistochemical assay of nucleoprotein viral antigen distribution provided the involvement of the respiratory tract only, but not gastrointestinal tracts. Thus suggesting that the fecal transmission of influenza virus type A H5N1 is not fully right. Further studies are necessary to elucidate the role of this respiratory organ in the pathobiology and transmission of influenza virus type A of both LPAI and HPAI.

### Materials and Methods

Nine apparently healthy domestic ducks (*Cairina moschata*) were collected from poultry farm in the rural area of Yogyakarta Special Province, Central Java, Indonesia. All the ducks had no clinical signs of influenza virus type A. The ducks were submitted for necropsy at the Department of Pathology, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta, Indonesia. Furthermore, the immunohistochemical streptavidin biotin and molecular analysis reverse transcriptase-polymerase chain reaction were done at the Immunology and Molecular Laboratory, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta, Indonesia.

The 100 mg of the lungs and tracheas tissues or the intestinal organs, including the pancreas tissues were homogenized using sterile tissue homogenizer and were subject to RNA extraction. RNA extraction and purification was done using commercial QIAmp Viral RNA extraction kit according to the instructions provided by the manufacturer (Qiagen, Chatsworth, California). The RNA was eluted in 50 microliters of RNase free H<sub>2</sub>O, and either used

immediately or stored at -70°C.

Oligonucleotide primers were synthesized by a commercial source (Integrated DNA Technologies Inc., Coralville, Iowa USA). The nucleotide sequences of PCR primers used in this study were as follows: NP - HW 63 : 5' - AGCAAAAGCAGGGTAGATAA -3' and NP-HW64 : 5'-TCC TTGCATCAGAGAGCA CA -3', H5-HW65: 5'-TATGCCTATAAAATTGTCAAG-3' and H5 - HW 66 : 5' - ATACCATCCATCTACCATTCC-3' and N1-HW71: 5'-TTGCTTGG TCAGCA AGTCCT-3' and N1-HW72: 5'-TCTGTCCATCCATTAGGATCC-3'. Amplification of each (NP, H5 and N1) viral gene was accomplished by using the SuperScript One-Step RT-PCR with Platinum Taq. (Invitrogen, USA). Thermocycling conditions for each gene consisted of reverse transcription step at 45°C-50°C for 30-45 min followed by denaturation at 95°C for 15 min and subsequently by 36-40 cycles of heat denaturation at 94°C for 30 sec., primer annealing at 52°C for 30 sec-1min., primer extension at 72°C for 1-2 min and a final extension step of 72°C for 7 min.

PCR products (5 microliters) were analyzed in 1.5% agarose gels in 1X TAE buffer, containing 0.1 micrograms/ml of ethidium bromide. The RT-PCR products were then analyzed by electrophoresis.

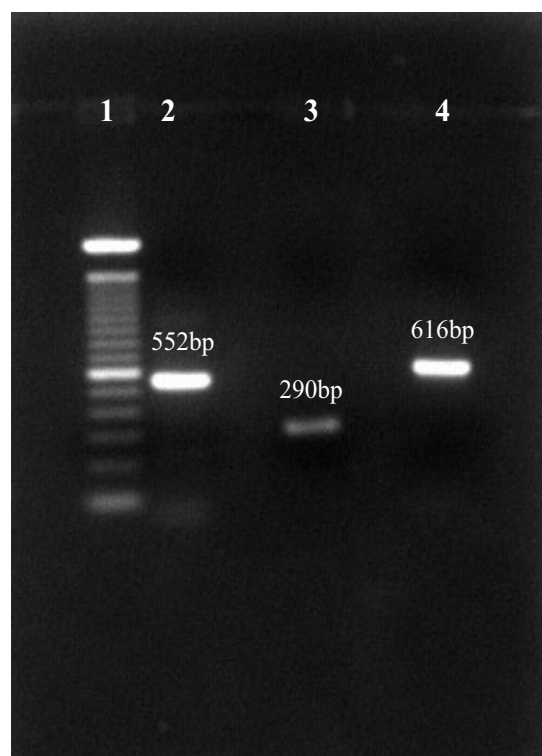
Lung and intestinal tissues for histopathologic evaluation was fixed by submersion in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Sections were made at 5 µm and was immunohistochemically stained using a mouse-derived monoclonal antibody (P13C11) specific for type A influenza virus nucleoprotein (NP) antigen as the primary antibody (SEPR, Athens, GA, USA). Rehydratedly histopathologic

sections were washed for 10 minutes in phosphate buffer saline (PBS; 0.01M, pH 7.6). The sections were kept for 10 minutes in 3% H<sub>2</sub>O<sub>2</sub> in absolute methanol to block endogenous peroxidase activity. After washing the sections in PBS for 10 minutes, non-specific antibody binding was suppressed by normal goat serum diluted 1:20 in 0.01 M PBS for 10 minutes. Then, the sections were incubated with monoclonal antibody anti-NP diluted 1:100 in 0.01 M PBS, pH 7.6 for 45 minutes at room temperature. After washing in PBS, the sections were incubated with biotinylated rabbit anti-mouse IgG for 10 minutes at room temperature. The sections were washed with PBS for 10 minutes. After washing in PBS, the bound antibody in the sections was visualized by the streptavidin-biotin-peroxidase

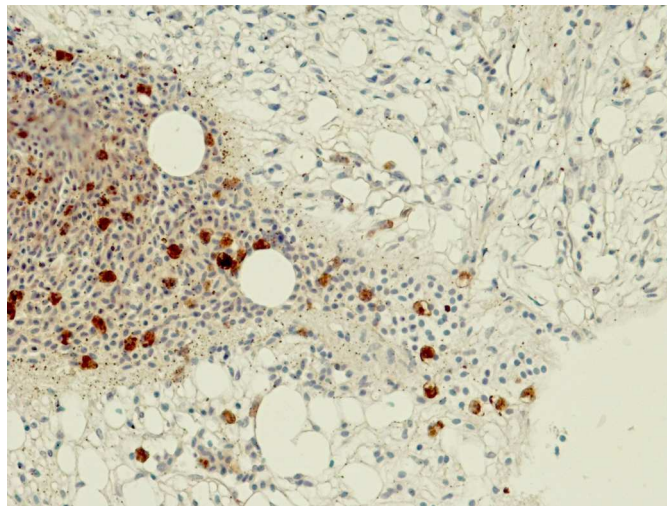
procedure using streptavidin biotin kit (Zymed, South San Francisco, CA, USA) and diaminobenzidine (DAB) as chromogen. Control sections incubated without the primary antibody, showed no specific cell staining.

## Results and Discussion

In this study, using the one tube RT-PCR technique, the avian influenza virus type A subtype H5N1 in the lungs and tracheas of the apparently healthy ducks were successfully amplified. The DNA bands from these organs were clearly visible at 552 bp (NP), 290 bp (H5) and 616 bp (N1) (Fig 1). Intestinal and pancreatic organs of the ducks samples considered to be negative.



**Figure 1.** One tube RT-PCR run on 1.5% agarose gel electrophoresis for influenza virus type A NP of 552 bp, H5 of 290 bp and N1 of 616 bp genes in the lungs and tracheas tissues. Lane 2: DNA marker 100 bp, Lane 4: NP positive, Lane 5: H5 positive and Lane 6: N1 positive



**Figure 2.** Immunohistochemical staining of formalin-fixed paraffin-embedded tissue of the lung tissue using monoclonal antibody against influenza virus type A nucleoprotein (NP). Positive staining for NP indicated by reddish brown in color. Virus located mostly within the vascular endothelium of the lung (Streptavidin biotin, 500x.).

The data presented in Figure 2 clearly shows that the immunohistochemical detection method of streptavidin biotin consistently detects influenza virus type A NP in the lungs tissues that were positive by that of on one tube RT-PCR.

Many domestic and wild avian species are infected with influenza virus type A. These include chickens, ducks, guinea fowl, domestic geese, quail, pheasant, partridge, psittacine, gulls, shorebirds (Easterday *et al.*, 1997; Webster and Kawaoka, 1988). Influenza virus type A produces an array of syndromes in birds, ranging from asymptomatic to mild upper respiratory infections to loss of egg production to rapidly fatal systemic disease (Easterday *et al.*, 1997). Most avian influenza viruses isolated in the field are a virulent. Virulent viruses have never been isolated from apparently healthy waterfowl, with the exception of pathogenic isolates collected from ducks or geese near a chicken influenza outbreak (Kawaoka *et al.*, 1987; Rohm *et*

*al.*, 1996). In fact, ducks are typically resistant to viruses that are lethal in chickens. For example, A/Turkey/Ireland/1378/85 (H5N8), which readily kills chickens and turkeys, does not cause disease symptoms in ducks (Kida *et al.*, 1980), even though it can be detected in a variety of internal organs and in the blood of infected birds (Kawaoka *et al.*, 1987). Influenza viruses are secreted from the intestinal tract into the feces of infected birds (Murphy and Webster, 1996; Webster *et al.*, 1992; Webster *et al.*, 1978).

In an effort to gain insight into the possible role of domestic ducks in Indonesia that may be acting as a silent reservoir for the influenza virus type A H5N1 to poultry and, possible to humans as well, the 9 apparently healthy domestic ducks from poultry farm in the rural area of Yogyakarta Special Province, Central Java, Indonesia which has never had any influenza virus type A H5N1 outbreak since 2003 were collected. In fact, although ducks are



considered to secrete the influenza virus from the intestinal tract into the feces (Horimoto and Kawaoka, 2001; Webster *et al.*, 1992), it appears that in the present study, one of the constant findings observed in all apparently healthy ducks is the involvement of the respiratory tract. The influenza virus type A H5N1 was identified from the respiratory tracts tissues, but not from the intestinal tracts tissues by RT-PCR. The gross lesions were also only observed in the lungs and the tracheas, but not in the intestines. The lungs were affected by moderate to severe diffuse petechial hemorrhages and the tracheas had mild to moderate linear hemorrhages. No gross lesions were detected in the intestines and the pancreas. Severe diffuse congestion and hemorrhages of pulmonary parenchyma were mainly observed histopathologically. Immunohistochemistry assays for influenza virus type A nucleoprotein (NP) antigen was positive in the lungs tissues examined. These findings implicate the tissue tropism of influenza virus type A H5N1 and suggest that differences in the tissue distribution of virus might determine the outcome of the virus transmission. Apparently, airborne transmission or direct contact with contaminated respiratory secretions essential for infectivity and for spread of the virus through the silent carrier of influenza virus type AH5N1.

Since an ancestral HPAI in chicken cases in Indonesia has not been isolated, these results may verify that the possible route of bird-to-bird transmission of the H5N1 virus to poultry is direct contact with contaminated respiratory secretion

from the ducks. The concern is greatest in rural HPAI affected areas where traditional free-ranging domestic ducks and chicken sharing the same source of littermates and of water. In addition, the 1997 Hong Kong HPAI outbreak in humans was unique in that it suggested additional models for the generation of pandemic strains from avian viruses, those are direct transmission and reassortment or adaptation (Fouchier *et al.*, 2004). Thus, further studies are necessary to elucidate the role of this respiratory tract organ of the apparently healthy ducks in the pathogenesis and transmission of HPAI on chickens, and, possible, on humans as well.

### Acknowledgments

We thank Dr. Roger K Maes, Professor of Virology, Michigan State University, East Lansing, MI, USA, for providing the molecular and immunological reagents.

### References

- Alexander, D.J. (2000) A review of avian influenza in different bird species. *Vet. Microbiol.* 74: 3-13.
- Alexander, D.J. (2001) Ecology of avian influenza in domestic birds. In: Dodet B, Vicari, M (eds.). *Emergence and Control of Zoonotic Ortho-and paramyxovirus Diseases*. John Libbey Eurotext, Paris, France. 25-33.
- Bosch, F.X., Garten, W., Klenk, H.D. and Rott, R. (1981) Proteolytic cleavage of influenza virus hemagglutinins: Primary structure of the connecting peptide between HA1 and HA2 determines proteolytic cleavability and pathogenicity of avian influenza viruses. *Virology* 113: 725-735.

- Easterday, B.C., Hinshaw, V.S., Halvorson, D.A. 1997. Influenza. In: Calnek, B.W., Barnes, H.J., Beard, C.W., McDougald, L.R. Disease of poultry. 10<sup>th</sup> ed.. Iowa State University Press, Ames, Iowa, USA.
- Fouchier, R.A.M., Schneeberger, P.M., Rozendaal, F.W., Broekman, J.M., Kemink, S.A., Munster, V., Kuiken, T., Rimmelzwaan, G.F., Schutten, M., Van Doormum, G.J., Koch, G., Bosman, A., Koopmans, M. and Osterhaus, A.D. (2004) Avian influenza A virus (H7N7) associated with conjunctivitis and a fatal case of acute respiratory distress syndrome. *Proc. Natl. Acad. Sci.* 101: 1356-1361.
- Fouchier, R.A.M., Munster, V. and Willemsen, A. (2005) Characterization of a novel influenza A virus haemagglutinin subtype (H16) obtained from black-headed gulls. *J. Virol.* 79: 2814-2822.
- Guan, Y., Peiris, M., Kong, K.F., Dyrting, K.C., Ellis, T.M., Sit, T., Zhang, I.J. and Shortridge, K.F. (2002) H5N1 influenza viruses isolated from geese in Southeastern China: Evidence for genetic reassortment and interspecies transmission to ducks. *Virology* 292: 16-23.
- Horimoto, T. and Kawaoka, Y. (2001) Pandemic threat posed by avian influenza A viruses. *Clin. Microbiol. Rev.* 14: 129-149.
- Kawaoka, Y., Nestorowicz, D.J., Alexander, D.J. and Webster, R.G. (1987) Molecular analyses of the hemagglutinin genes of H5 influenza viruses origin of a virulent turkey strain. *Virology* 158: 218-227.
- Kida, H., Yanagawa, R. and Matsuoka, Y. (1980) Duck influenza lacking evidence of disease signs and immune response. *Infect. Immun.* 30: 547-553.
- Murphy, B.R. and Webster, R.G. (1996) *Orthomyxoviruses* In: B.N. Fields, D.M. Knipe, and P.M. Howley (Ed). 3<sup>rd</sup> ed. Vol. 1. Fields Virology, Lippincott-Raven Publisher, Philadelphia, Pa, USA.: 1397-1445.
- Rohm, C., Suss, J., Pohle, V. and Webster, R.G. (1996) Different hemagglutinin cleavage site variants of H7N7 in an influenza outbreak in chickens in Leipzig, Germany. *Virology* 218: 253-257.
- Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M. and Kawaoka, Y. (1992) Evolution and ecology of influenza A viruses. *Microbiol. Rev.* 56: 152-179.
- Webster, R.G. and Kawaoka, Y. (1988) Avian influenza. *Crit. Rev. Poult. Biol.* 1: 211-246.
- Webster, R.G., Yakhno, M.A., Hinshaw, V.S., Bean, W.J. and Murti, G.K. (1978) Intestinal influenza: Replication and characterization of influenza viruses in ducks. *Virol.* 84: 268-278.